Method for Increasing Luminescence Assay Sensitivity

Field of the Invention

The present invention relates generally to the fields of cell biology and molecular biology. In particular, this invention relates to methods, compositions and kits for increasing the sensitivity of a luminescence assay measurement.

Background of the Invention

Reporter molecules are routinely used to monitor molecular events in the fields of biology, biochemistry, immunology, cell biology and molecular biology. For example, reporter molecules are employed in assays where the levels of the reporter molecule are due to transcription from a specific promoter linked to the reporter molecule. These assays can be used to study eukaryotic gene expression, receptor activity, transcription factors, intracellular signaling, mRNA processing, protein folding, and the like. Reporter molecules that are typically used in such assays include radioactive isotopes, fluorescent agents, enzymes, and luminescent agents. See for example, Akhavan-Tafti, et al, in: Bioluminescence and Chemiluminescence. Fundamentals and Applied Aspects. Proceedings of the 8th International Symposium on Bioluminescence and Chemiluminescence. Cambridge, September 1994. Eds. Campbel, Kricka, Stanley. John Wiley and Sons 1994.

Two luminescent enzymes that are particularly useful in assay systems are firefly luciferase and *Renilla reniformis* luciferase. The substrates for these luciferases and the reaction products they produce are shown in Figures 1 and 2. The quantity of light (i.e. the number of photons) produced in the reaction, can be measured and used to calculate the concentration of luminescent enzyme in the reaction.

Firefly luciferase is a 61 kDa monomeric protein that does not require post-translational processing for enzymatic activity. Thus, it functions as a

genetic reporter immediately upon translation. Photon emission is achieved through oxidation of beetle luciferin in a reaction that requires ATP, Mg^{2+} and O_2 (Figure 1).

Renilla luciferase is a 36 kDa monomeric protein that is composed of 3% carbohydrate when purified from its natural source, *Renilla reniformis*. Like firefly luciferase, post-translational modification of *Renilla* luciferase is not required for its activity, and it functions as a genetic reporter immediately following translation. The luminescent reaction catalyzed by *Renilla* luciferase utilizes O_2 and coelenterate-luciferin, also called coelenterazine (Figure 2).

Luminescent reactions can be used to detect very small quantities of a particular analyte, the substance being identified and measured in an analysis. For example, luminescent reactions can be used to detect and quantify proteases, lipases, phosphatases, peroxidases, glycosidases, and various metabolites such as ATP or NADH. Luminescent reactions can also be used to detect and quantify analytes through binding interactions, such as those mediated by antibodies and nucleotide probes. Typically, luminescent reactions can be used to detect less than $1x10^{-16}$ moles of analyte in a sample, often less than $1x10^{-19}$ moles. In luminescence, commonly detected analytes are the luciferases, especially firefly luciferase and *Renilla* luciferase. Most often these analytes are used to quantify phenomena associated with their creation through gene expression and protein synthesis. Other luminescent enzymes used as analytes include, but are not limited to, aequorin, *Vargula* luciferase, and other marine luciferases.

When using luminescence to measure an analyte, it is preferred that little or no light is produced by reactions that are not dependent on the presence of the analyte. This is the case with firefly luciferase. Under typical firefly luciferase assay conditions, luminescence cannot be detected when the firefly luciferase is not present. In contrast to assays employing firefly luciferase, light can generally be detected in *Renilla* luciferase assay systems when the *Renilla* luciferase is not present. Luminescence that is not dependent on the catalytic activity of a luminescent enzyme is termed autoluminescence. For example, autoluminescence can be caused by spontaneous oxidation of the luminogenic substrate coelenterazine.

Luminescence that is not dependent on the on the presence of an analyte (e.g. autoluminescence) can limit the usefulness of an analytical assay by reducing the ability to accurately measure the quantity of light resulting from the activity of the analyte. In particular, the sensitivity of luminescent assays containing coelenterazine or its structural analogs is reduced due to autoluminescence. Additionally, the addition of various components to the assay system, such as lipids (especially above the critical micelle concentration or CMC), hydrophobic proteins (especially those with a defined three-dimensional structure), and cells or other biological materials containing hydrophobic microenvironments, can greatly increase autoluminescence.

Assay sensitivity may also be reduced by luminescence from an unrelated luminogenic molecule. The unrelated luminogenic molecule may be present due to contamination of the analytical assay, or due to a separate analytical luminescence assay performed in the same reaction mixture. In either case, the sensitivity of an analytical luminescence assay could be improved by reducing the luminescence that is not dependent on the presence of the analyte.

Summary of the Invention

Applicants have discovered that the sensitivity of luminescence assays can be improved by carrying out the assay in the presence of one or more organic compounds that reduce analyte-independent luminescence. In particular, Applicant has unexpectedly discovered that the analyte-independent luminescence can be reduced without similarly reducing analyte-dependent luminescence. Preferably, the analyte-dependent luminescence is reduced by a lower fold than the analyte-independent luminescence, or the analyte dependent luminescence remains about the same or increases. Accordingly, the invention provides a method for increasing the sensitivity of a luminescent assay comprising carrying out the assay in the presence of an organic compound that reduces luminescence that is not dependent on the presence of an analyte by a factor of at least about 10 fold, and that

reduces luminescence that is dependent on the presence of an analyte by less than about 7 fold.

The invention also provides a method for increasing the sensitivity of a luminescent assay comprising carrying out the assay in the presence of an organic compound that reduces luminescence generated by luminogenic molecules not bound to an enzyme by at least about 10 fold, and that reduces the luminescence generated by luminogenic molecules bound to an enzyme by less than about 7 fold.

The invention also provides a method for increasing the sensitivity of a luminescent assay comprising carrying out the assay in the presence of an organic compound that reduces autoluminescence by at least about 10 fold, and that reduces luminescence that is dependent on the presence of an analyte by less than about 7 fold.

The invention also provides a method for increasing the sensitivity of a luminescent assay comprising carrying out the assay in the presence of an organic compound that comprises a selenium atom.

The invention also provides a method for increasing the sensitivity of a luminescent assay comprising carrying out the assay in the presence of an organic compound that comprises a carbon-selenium bond.

The invention also provides a method for increasing the sensitivity of a luminescent assay comprising carrying out the assay in the presence of an organic compound that comprises a carbon selenium double bond (C=Se).

The invention also provides a method for increasing the sensitivity of a luminescent assay comprising carrying out the assay in the presence of an organic compound that comprises a carbon-selenium single bond (C-Se)

The invention also provides a method for increasing the sensitivity of a luminescent assay comprising carrying out the assay in the presence of an organic compound that comprises a carbon-sulfur double bond (C=S).

The invention also provides a method for increasing the sensitivity of a luminescent assay comprising carrying out the assay in the presence of an organic

compound that comprises a carbon atom bound to both a selenium atom and a nitrogen atom.

The invention also provides a method for increasing the sensitivity of a luminescent assay comprising carrying out the assay in the presence of an organic compound that comprises a carbon atom bound to both a sulfur atom and a nitrogen atom.

The invention also provides a method for increasing the sensitivity of a luminescent assay comprising carrying out the assay in the presence of an organic compound that comprises a sulfur atom bound to two carbon atoms, wherein the analyte-independent luminescence is reduced by at least about 10 fold. Preferably, the analyte-dependent luminescence is reduced by less than 7 fold.

The invention also provides an assay kit comprising packaging material containing 1) a luminogenic substrate of a luminescent enzyme, or a luminogenic enzyme; and 2) an organic compound for increasing the sensitivity of a luminescent assay. Preferably, the organic molecule is capable of 1) reducing the luminescence that is not dependent on the presence of an analyte by a factor of at least about 10 fold, and reducing the luminescence that is dependent on the presence of an analyte by less than about 7 fold; 2) reducing the luminescence generated by luminogenic molecules not bound to an enzyme by at least about 10 fold, and reducing the luminescence generated by luminogenic molecules bound to an enzyme by less than about 7 fold; or 3) reducing autoluminescence by at least about 10 fold, and reducing luminescence that is dependent on the presence of an analyte by less than about 7 fold.

The invention also provides novel compounds disclosed herein that are useful to increase the sensitivity of a luminescent assay.

Brief Description of the Figures

FIG. 1 illustrates chemiluminescent reaction catalyzed by firefly luciferase.

FIG. 2 illustrates chemiluminescent reaction catalyzed by *Renilla* luciferase.

FIG. 3 illustrates a dioxetane intermediate in the colenterazine autoluminescence pathway.

FIG. 4 shows representative compounds (1-11) that reduce autoluminescence.

Detailed Description

Before the present invention is disclosed and described in detail, it is to be understood that this invention is not limited to specific assay formats, materials or reagents, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

As used in the specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings, unless otherwise described:

The term "halo" as used herein denotes fluoro, chloro, bromo, or iodo.

The terms "Alkyl", "alkoxy", "alkenyl", "alkynyl", etc. as used herein denote both branched and unbranched groups; but reference to an individual radical such as "propyl" embraces only the straight, unbranched chain radical, a branched chain isomer such as "isopropyl" being specifically referred to.

The term "Aryl", as used herein, denotes a monocyclic or polycyclic hydrocarbon radical comprising 6 to 30 atoms wherein at least one ring is aromatic. Preferably, aryl denotes a phenyl radical or an ortho-fused bicyclic carbocyclic radical having about nine to ten ring atoms in which at least one ring is aromatic. "Heteroaryl" encompasses a radical of a monocyclic aromatic ring containing five or six ring atoms consisting of carbon and one to four heteroatoms each selected from

the group consisting of non-peroxide oxygen, sulfur, and N(X) wherein X is absent or is H, O, (C_1-C_4)alkyl, phenyl or benzyl, as well as a radical of a polycyclic ring comprising 8 to 30 atoms derived therefrom. Preferably, heteroaryl encompasses a radical attached via a ring carbon of a monocyclic aromatic ring containing five or six ring atoms consisting of carbon and one to four heteroatoms each selected from the group consisting of non-peroxide oxygen, sulfur, and N(X) wherein X is absent or is H, O, (C_1-C_4)alkyl, phenyl or benzyl, as well as a radical of an ortho-fused bicyclic heterocycle of about eight to ten ring atoms derived therefrom, particularly a benz-derivative or one derived by fusing a propylene, trimethylene, or tetramethylene diradical thereto.

The term "analyte", as used herein is a substance to be detected in a test sample. In luminescence assays, commonly detected analytes include the luciferases, especially firefly luciferase and *Renilla* luciferase. Other luminescent enzymes used as analytes include, but are not limited to, aequorin, *Vargula* luciferase, and other marine luciferases. Additionally, luminescent reactions can be used to detect and quantify analytes such as proteases, lipases, phosphatases, peroxidases, glycosidases, and various metabolites such as ATP or NADH. Luminescent reactions can also be used to detect and quantify analytes through binding interactions, such as those mediated by antibodies and nucleotide probes. In certain cases, analyte-dependent luminescence can be coupled to the activity of a luminescent enzyme. For example, alkaline phosphatase (AP) could be detected by using a phospho derivative of luciferin. By this strategy, luciferin is generated by the action of AP, which then yields light by reaction with luciferase. The instant invention would allow the AP assay to be run after a separate horseradish peroxidase/luminol reaction. With respect to the analyte AP, a compound as described herein, could be added to reduce the analyte-independent luminescence caused by horseradish peroxidase.

The term "autoluminesence" as used herein, refers to the release of light from a luminogenic molecule that does not result from enzymatic action on the luminogenic molecule.

The term "increase the sensitivity of a luminescent assay" as used herein means increasing the precision of the assay or improving the ability to measure the presence of a small amount of an analyte with the assay. For example, the sensitivity of a luminescent assay can be increased by reducing analyte-independent luminescence. Preferably, analyte-independent luminescence is reduced, and a minimal reduction, no reduction, or an increase in analyte-dependent luminescence results. Additionally, analyte-independent luminesence is preferably reduced by a greater fold than analyte-dependent luminescence.

The term "luminescent," as used herein, includes bio-luminescence (i.e light produced by a living organism), chemi-luminescence (light produced when a chemical reaction proceeds), and electrochemical-luminescence. When the enzyme involved has evolved in an organism by natural selection for the purpose of generating light, or the enzyme involved is a mutated derivative of such an enzyme, the luminescent reactions are also called "bioluminescent reactions" and the enzyme involved is also called a "bioluminescent enzyme." Examples are firefly luciferase, *Renilla* luciferase, Cypridina luciferase, Aequorin photoprotein, Obelin photoprotein, and the like.

The term "luminescent assay" or "luminescence assay" includes any assay that generates light based on the presence of an analyte. Such assays include assays that employ one or more luciferase enzymes (e.g. firefly luciferase, *Renilla* luciferase, *Cypridina* luciferase, and the like).

The term "luminogenic enzyme," as used herein includes enzymes that catalyze a reaction that produces light, or that lead to the production of light. For example, the term includes firefly luciferase, *Renilla* luciferase, Cypridina luciferase, Aequorin photoprotein, Obelin photoprotein, and the like.

The term "luminogenic molecule" as used herein refers to a molecule capable of creating light via a chemical reaction (e.g. luciferin, coelenterazine, or a functional analog thereof). Generally, a luminogenic molecule is either a high energy molecular species (e.g. a stabilized dioxetane), or it is transformed into a high energy molecular species by a chemical reaction. The chemical reaction is

usually oxidation by oxygen, superoxide, or peroxide. In each case, the energy within the luminogenic molecule is released by the chemical reaction. Although at least some of this energy is released as photons of light, the energy can also be released in other forms, such as heat. The luminogenic molecules that do not yield light disperse their energy through alternative modes, often termed "dark pathways".

The term "luminogenic molecule not bound to an enzyme" as used herein, includes a luminogenic molecule that is not bound to an enzyme (e.g. firefly luciferase, *Renilla* luciferase, *Cypridina* luciferase, and the like) that catalyzes a reaction that produces light.

The term "luminogenic molecules bound to an enzyme" as used herein includes a luminogenic molecule that is bound to an enzyme that catalyzes a reaction that produces light.

The term "luminescence that is dependent on the presence of an analyte," or "analyte-dependent luminescence" as used herein, includes luminescence that results from a chemical reaction that involves an analyte, as well as luminescence that correlates with the presence of an analyte either directly or indirectly.

The term "luminescence that is not dependent on the presence of an analyte," or "analyte-independent luminescence" as used herein, includes luminescence resulting from autoluminescence of a luminogenic substrate as well as luminescence resulting from an unrelated luminogenic molecule present in an assay mixture.

The term "quench" as used herein means to reduce the yield of photons from a luminescent reaction. The term includes preventing an analyte from being detected or being detectable, and may occur either directly or indirectly. Agents that can be used to quench a reaction are known as "quenching agents."

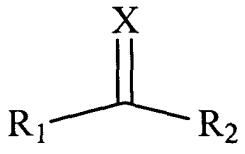
Applicant has discovered that it is possible to increase the sensitivity of a luminescent assay by carrying out the assay in the presence of an organic compound that reduces analyte-independent luminescence. This finding is unexpected. Using procedures similar to those described herein, one skilled in the

art can identify compounds that are suitable for increasing the sensitivity of a luminescent assay. The structure of the compound is not critical provided the compound is capable of increasing the sensitivity of a luminescent assay.

In particular, applicant has discovered that compounds that comprise a sulfur atom or a selenium atom are particularly useful for increasing the sensitivity of a luminescent assay. The remaining chemical structure of the compound that comprises a selenium atom or a sulfur atom is not critical, provided the structure does not interfere with the function of the compound. Preferred compounds have low toxicity at concentrations used in the invention, and can be stored, transported, and disposed of inexpensively.

Suitable compounds include organic compounds (i.e. compounds that comprise one or more carbon atoms). Suitable organic compounds can comprise a carbon-sulfur bond or a carbon-selenium bond, for example suitable organic compounds can comprise a carbon-sulfur double bond (C=S), a carbon selenium double bond (C=Se), a carbon-sulfur single bond (C-S), or carbon-selenium single bond (C-Se). Suitable organic compounds can also comprise a carbon bound mercapto group (C-SH) or a sulfur atom bound to two catbon atoms (C-S-C). Preferred compounds are lipophyllic in nature.

Suitable compounds that comprise a carbon sulfur double bond or a carbon selenium double bond include for example compounds of formula (I):



wherein X is S or Se; R_1 and R_2 are each independently hydrogen, $(C_1\text{-}C_{20})$alkyl, $(C_3\text{-}C_8)$cycloalkyl, $(C_1\text{-}C_{20})$alkoxy, $(C_2\text{-}C_{20})$alkenyl, $(C_2\text{-}C_{20})$alkynyl, aryl, heteroaryl, or NR_aR_b; or R_1 and R_2 together with the carbon to which they are attached form a 5, 6, 7, or 8 membered saturated or unsaturated ring comprising carbon and optionally comprising 1, 2, or 3 heteroatoms selected from oxy (-O-), thio (-S-), or nitrogen (-NR_c)-, wherein said ring is optionally substituted with 1, 2, or 3 halo, hydroxy, oxo,

thioxo, carboxy, (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl, (C_1-C_{20})alkoxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, (C_2-C_{20})alkenyl, (C_2-C_{20})alkynyl, aryl, or heteroaryl; and R_a, R_b and R_c are each independently hydrogen, (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl,(C_2-C_{20})alkenyl, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, (C_2-C_{20})alkynyl, aryl, heteroaryl; wherein any (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl, (C_1-C_{20})alkoxy, (C_2-C_{20})alkenyl (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, or (C_2-C_{20})alkynyl of R_1 , R_2 , R_a, R_b, and R_c is optionally substituted with one or more (e.g 1, 2, 3, or 4) halo, hydroxy, mercapto, oxo, thioxo, carboxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, aryl, or heteroaryl; and wherein any aryl or heteroaryl is optionally substituted with one or more (1, 2, 3, or 4) halo, hydroxy, mercapto, carboxy, cyano, nitro, trifluoromethyl, trifluoromethoxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkanoyloxy, sulfo or (C_1-C_{20})alkoxycarbonyl; or a salt thereof.

Suitable compounds that comprise a mercapto group include for example compounds of the formula R_3SH wherein: R_3 is (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl,(C_2-C_{20})alkenyl, (C_2-C_{20})alkynyl, aryl, or heteroaryl; wherein any (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl, (C_2-C_{20})alkenyl, or (C_2-C_{20})alkynyl of R_3 is optionally substituted with one or more (e.g 1, 2, 3, or 4) halo, hydroxy, mercapto oxo, thioxo, carboxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, aryl, heteroaryl, or NR_dR_e; wherein R_d and R_e are each independently hydrogen, (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl,(C_2-C_{20})alkenyl, (C_2-C_{20})alkynyl, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl aryl, or heteroaryl; and wherein any aryl or heteroaryl is optionally substituted with one or more (1, 2, 3, or 4) halo, mercapto, hydroxy, oxo, carboxy, cyano, nitro, trifluoromethyl, trifluoromethoxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkanoyloxy, sulfo or (C_1-C_{20})alkoxycarbonyl; or a salt thereof.

Other suitable compounds include for example compounds of the formula R_4NCS wherein: R_4 is (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl,(C_2-C_{20})alkenyl, (C_2-C_{20})alkynyl, aryl, or heteroaryl; wherein any (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl, (C_2-C_{20})alkenyl, or (C_2-C_{20})alkynyl of R_3 is optionally substituted with one or more (e.g 1, 2, 3, or 4) halo, hydroxy, mercapto oxo, thioxo, carboxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, aryl, heteroaryl, or NR_fR_g; wherein R_f and R_g are each

independently hydrogen, (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl,(C_2-C_{20})alkenyl, (C_2-C_{20})alkynyl, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl aryl, or heteroaryl; and wherein any aryl or heteroaryl is optionally substituted with one or more (1, 2, 3, or 4) halo, mercapto, hydroxy, oxo, carboxy, cyano, nitro, trifluoromethyl, trifluoromethoxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkanoyloxy, sulfo or (C_1-C_{20})alkoxycarbonyl; or a salt thereof

Other suitable compounds that comprise a carbon-selenium single bond or a carbon sulfur single bond include compounds of formula R_5-X-R_6 wherein:

X is -S- or -Se-;

R_5 is (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl,(C_2-C_{20})alkenyl, (C_2-C_{20})alkynyl, aryl, or heteroaryl; and R_6 is hydrogen, (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl,(C_2-C_{20})alkenyl, (C_2-C_{20})alkynyl, aryl, or heteroaryl;

or R_5 and R_6 together with X form a heteroaryl;

wherein any (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl, (C_2-C_{20})alkenyl, or (C_2-C_{20})alkynyl of R_5 or R_6 is optionally substituted with one or more (e.g 1, 2, 3, or 4) halo, hydroxy, mercapto oxo, thioxo, carboxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, aryl, heteroaryl, or NR_kR_m;

wherein R_k and R_m are each independently hydrogen, (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl,(C_2-C_{20})alkenyl, (C_2-C_{20})alkynyl, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl aryl, or heteroaryl; and

wherein any aryl or heteroaryl is optionally substituted with one or more (1, 2, 3, or 4) halo, mercapto, hydroxy, oxo, carboxy, cyano, nitro, trifluoromethyl, trifluoromethoxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkanoyloxy, sulfo or (C_1-C_{20})alkoxycarbonyl; or a salt thereof.

Specific and preferred values listed below for radicals, substituents, and ranges, are for illustration only; they do not exclude other defined values or other values within defined ranges for the radicals and substituents

Specifically, (C_1-C_{20})alkyl can be methyl, ethyl, propyl, isopropyl, butyl, iso-butyl, sec-butyl, pentyl, 3-pentyl, or hexyl; (C_3-C_8)cycloalkyl can be

cyclopropyl, cyclobutyl, cyclopentyl, or cyclohexyl; (C_1-C_{20})alkoxy can be methoxy, ethoxy, propoxy, isopropoxy, butoxy, iso-butoxy, sec-butoxy, pentoxy, 3-pentoxy, or hexyloxy; (C_2-C_{20})alkenyl can be vinyl, allyl, 1-propenyl, 2-propenyl, 1-butenyl, 2-butenyl, 3-butenyl, 1,-pentenyl, 2-pentenyl, 3-pentenyl, 4-pentenyl, 1- hexenyl, 2-hexenyl, 3-hexenyl, 4-hexenyl, or 5-hexenyl; (C_2-C_{20})alkynyl can be ethynyl, 1-propynyl, 2-propynyl, 1-butynyl, 2-butynyl, 3-butynyl, 1-pentynyl, 2-pentynyl, 3-pentynyl, 4-pentynyl, 1- hexynyl, 2-hexynyl, 3-hexynyl, 4-hexynyl, or 5-hexynyl; (C_1-C_{20})alkanoyl can be acetyl, propanoyl or butanoyl; (C_1-C_{20})alkoxycarbonyl can be methoxycarbonyl, ethoxycarbonyl, propoxycarbonyl, isopropoxycarbonyl, butoxycarbonyl, pentoxycarbonyl, or hexyloxycarbonyl; (C_2-C_{20})alkanoyloxy can be acetoxy, propanoyloxy, butanoyloxy, isobutanoyloxy, pentanoyloxy, or hexanoyloxy; aryl can be phenyl, indenyl, or naphthyl; and heteroaryl can be furyl, imidazolyl, triazolyl, triazinyl, oxazoyl, isoxazoyl, thiazolyl, isothiazoyl, pyrazolyl, pyrrolyl, pyrazinyl, tetrazolyl, pyridyl, (or its N-oxide), thienyl, pyrimidinyl (or its N-oxide), indolyl, isoquinolyl (or its N-oxide) or quinolyl (or its N-oxide).

Specifically, R_1 and R_2 can each independently be hydrogen, (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl,(C_2-C_{20})alkenyl, (C_2-C_{20})alkynyl, aryl, heteroaryl, or NR_aR_b; wherein R_a and R_b are each independently hydrogen, (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl,(C_2-C_{20})alkenyl, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, (C_2-C_{20})alkynyl, aryl, or heteroaryl; wherein any (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl, (C_1-C_{20})alkoxy, (C_2-C_{20})alkenyl (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, or (C_2-C_{20})alkynyl of R_1 , R_2 , R_a, and R_b is optionally substituted with 1 or 2 halo, hydroxy, mercapto, oxo, thioxo, carboxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, aryl, or heteroaryl; and wherein any aryl or heteroaryl is optionally substituted with one or more halo, hydroxy, mercapto, carboxy, cyano, nitro, trifluoromethyl, trifluoromethoxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkanoyloxy, sulfo or (C_1-C_{20})alkoxycarbonyl.

Specifically, R_1 and R_2 can each independently be hydrogen, (C_1-C_{10})alkyl,(C_2-C_{10})alkenyl, (C_2-C_{10})alkynyl, aryl, or NR_aR_b.

Specifically, R_1 and R_2 together with the carbon to which they are attached can form a 5 or 6 membered saturated or unsaturated ring comprising carbon and optionally comprising 1 or 2 heteroatoms selected from oxy (-O-), thio (-S-), or nitrogen (-NR_c)-, wherein said ring is optionally substituted with 1, 2, or 3 halo, hydroxy, oxo, thioxo, carboxy, (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl, (C_1-C_{20})alkoxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, (C_2-C_{20})alkenyl, (C_2-C_{20})alkynyl, aryl, or heteroaryl; wherein R_c is hydrogen, (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl,(C_2-C_{20})alkenyl, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, (C_2-C_{20})alkynyl, aryl, heteroaryl; wherein any (C_1-C_{20})alkyl, (C_3-C_{20})cycloalkyl, (C_1-C_{20})alkoxy, (C_2-C_{20})alkenyl (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, or (C_2-C_{20})alkynyl of R_1 , R_2 , and R_c is optionally substituted with one or more halo, hydroxy, mercapto, oxo, thioxo, carboxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, aryl, or heteroaryl; and wherein any aryl or heteroaryl is optionally substituted with one or more halo, hydroxy, mercapto, carboxy, cyano, nitro, trifluoromethyl, trifluoromethoxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkanoyloxy, sulfo or (C_1-C_{20})alkoxycarbonyl.

Specifically, R_1 and R_2 can each independently be NR_aR_b; wherein R_a and R_b are each independently hydrogen, (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl, (C_2-C_{20})alkenyl, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, (C_2-C_{20})alkynyl, aryl, heteroaryl; wherein any (C_1-C_{20})alkyl, (C_3-C_8)cycloalkyl, (C_2-C_{20})alkenyl (C_1-C_{20})alkanoyl, (C_1-C_{20})alkoxycarbonyl, or (C_2-C_{20})alkynyl is optionally substituted with one or more halo, hydroxy, mercapto, oxo, thioxo, carboxy, aryl, or heteroaryl; and wherein any aryl or heteroaryl is optionally substituted with one or more halo, hydroxy, mercapto, carboxy, cyano, nitro, trifluoromethyl, trifluoromethoxy, (C_1-C_{20})alkanoyl, (C_1-C_{20})alkanoyloxy, sulfo or (C_1-C_{20})alkoxycarbonyl.

Specifically, R_1 and R_2 can each independently be amino, (C_1-C_{20})alkyl, (C_1-C_{20})alkylamino, allylamino, 2-hydroxyethylamino, phenylamino, or 4-thiazoylamino.

Specifically, R_1 and R_2 can each independently be amino, methyl, allylamino, 2-hydroxyethylamino, phenylamino, or 4-thiazoylamino.

A specific value for R_3 is $(C_1$-$C_{20})$alkyl optionally substituted with one or more halo, mercapto oxo, thioxo, carboxy, $(C_1$-$C_{20})$alkanoyl, $(C_1$-$C_{20})$alkoxycarbonyl, aryl, heteroaryl, or NR_dR_e.

A specific value for R_3 is 2-aminoethyl, 2-amino-2-carboxyethyl, or 2-acylamino-2-carboxyethyl.

A specific value for R_4 is aryl, optionally substituted with one or more halo, mercapto, hydroxy, oxo, carboxy, cyano, nitro, trifluoromethyl, trifluoromethoxy, $(C_1$-$C_{20})$alkanoyl, $(C_1$-$C_{20})$alkanoyloxy, sulfo or $(C_1$-$C_{20})$alkoxycarbonyl.

Specifically, R_5 is $(C_1$-$C_{10})$alkyl, $(C_3$-$C_6)$cycloalkyl,$(C_2$-$C_{10})$alkenyl, $(C_2$-$C_{10})$alkynyl, aryl, or heteroaryl; and R_6 is hydrogen, $(C_1$-$C_{10})$alkyl, $(C_3$-$C_6)$cycloalkyl,$(C_2$-$C_{10})$alkenyl, $(C_2$-$C_{10})$alkynyl, aryl, or heteroaryl.

Specifically, R_5 and R_6 together with X form a heteroaryl.

Preferred organic compounds exclude polypeptides and proteins comprising one or more mercapto (C-SH) groups.

Preferred organic compounds exclude compounds that comprise one or more mercapto (C-SH) groups.

A preferred organic compound is a compound of formula 1-11 as shown in figure 4. A more preferred compound is thiourea.

The compounds described hereinabove are available from commercial sources or can be prepared from commercially available starting materials using procedures that are known in the field of synthetic chemistry. For example, see Jerry March, Advanced Organic Chemistry, 4th ed. Wiley-Interscience, John Wiley and Sons, New York, 1992.

In cases where compounds are sufficiently basic or acidic to form stable salts, use of the compounds as salts in the methods of the invention may be appropriate. Examples of suitable salts include organic acid addition salts, for example, tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate, α-ketoglutarate, and α-glycerophosphate salts. Suitable

inorganic salts may also be formed, including hydrochloride, sulfate, nitrate, bicarbonate, and carbonate salts.

Salts can be obtained using standard procedures well known in the art, for example by reacting a sufficiently basic compound with a suitable acid. Alkali metal (for example, sodium, potassium or lithium) or alkaline earth metal (for example calcium) salts can also be used.

When used in accord with the methods of the invention, the compounds described herein can be present in a luminescence reaction at any concentration that increases the sensitivity of the assay. The optimum concentration of a given compound will depend on the luminescent reagent(s) employed, and on the specific conditions under which a given assay is carried out. However, suitable concentrations can be determined using standard techniques that are available in the art.

Specifically, the compound that can increase the sensitivity of the assay can be present in a luminescence reaction at a concentration of at least about 0.1μM, or at a concentration of at least about 0.1mM. More specifically, the compound can be present in the luminescence reaction at a concentration in the range from about 0.1μM to about 500 mM (inclusive), or in the range from about 1μM to about 250 mM (inclusive). Preferably, the compound is present at a concentration in the range from about 10 μM to about 100 mM (inclusive).

Specifically, the assay can be performed in the presence of whole cells.

Specifically, the assay can be carried out in a solvent comprising at least about 10% water. More specifically, the invention can be carried out in a solvent comprising at least about 25% water, or at least about 40% water.

Preferably, in the practice of the methods of the invention, the analyte-independent luminescence is reduced by at least about 10 fold, or more preferably by at least about 20 fold, at least about 50 fold, or at least about 100 fold in the present of a compound, while the analyte-dependent luminescence is reduced by less than about 7 fold, about 5 fold, about 3 fold, or about 2 fold. For example, a

relative light unit value of 5 produced in the presence of the compound while a relative light unit value of 100 produced in the absence of the compound reflects a decrease in luminescence in the presence of the compound by 20 fold.

Preferably in the practice of the methods of the invention, the luminescence generated by luminogenic molecules not bound to an enzyme is reduced by at least about 10 fold, or more preferably by at least about 20 fold, at least about 50 fold, or at least about 100 fold, while the luminescence generated by luminogenic molecules bound to an enzyme is reduced by less than about 7 fold, about 5 fold, about 3 fold, or about 2 fold. The luminescence generated by luminonogenic molecules bound to an enzyme is preferably reduced by a lower fold than the fold decrease in luminescence generated by luminogenic molecules not bound to an enzyme.

Preferably in the practice of the methods of the invention, autoluminescence is reduced by at least about 10 fold, or more preferably by at least about 20 fold, at least about 50 fold, or at least about 100 fold, while luminescence that is dependent on the presence of an analyte is reduced by less than about 7 fold, about 5 fold, about 3 fold, or about 2 fold. The luminescence that is dependent on the presence of an analyte is preferably reduced by a lower fold than the fold decrease in autoluminescence.

Preferably in the practice of the methods of the invention, when an assay is carried out in the presence of a compound that comprises a sulfur atom or a selenium atom, analyte-independent luminescence is reduced by at least about 10 fold, or more preferably by at least about 20 fold, at least about 50 fold, or at least about 100 fold.

Preferably in the practice of the methods of the invention, when an assay is carried out in the presence of a compound that comprises a sulfur atom or a selenium atom, analyte-dependent luminescence is reduced by less than about 7 fold, about 5 fold, about 3 fold, or about 2 fold.

For kits of the invention the enzyme substrate, enzyme, and the compound can each be contained in a separate container, or they can be contained in

a single container. The kit can optionally comprise a buffer solution suitable for use in a luminescent assay, and the enzyme substrate or enzyme, and the buffer solution can optionally be contained in a single container. Additionally, the compound and the buffer solution can optionally be contained in a single container. The kits can also optionally comprise a second substrate (e.g. a substrate for firefly luciferase or *Renilla* luciferase), or a quenching agent for a luminescent enzyme reaction. The kits can also optionally comprise ATP, or can optionally comprise both a luminogenic substrate of a luminescent enzyme, and a luminogenic enzyme.

The ability of a compound to increase the sensitivity of a luminescent assay can be determined using assays that are well known to the art, or using the assays described in the Examples herein below.

Compounds identified herein have been shown to be useful for increasing the sensitivity of luminescent assays. The compounds are particularly useful for reducing luminescence that results from the decomposition of intermediate dioxetane rings. Thus, in addition to being useful for increasing the sensitivity of luminescent assays (e.g. bioluminescent, chemiluminescent, or electroluminescent assays), the compounds are also useful for reducing luminescence in other systems that involve intermediate dioxetane rings and the like.

The invention will now be illustrated by the following non-limiting Examples. Compounds 1-11 (Figure 4) are readily available from commercial sources.

Example 1. Improved assay sensitivity

Experiments to assess the ability of representative organic compounds ("compounds") to increase luminescence assay sensitivity were performed under the conditions described below in Format A and Format B. Improved luminescence assay sensitivity is demonstrated by the ability of the compounds to decrease the analyte-independent luminescence resulting from the oxidation of coelenterazine, while causing lesser or minimal reduction to the

analyte-dependent luminescence, i.e. coelenterazine in the presence of *Renilla* luciferase. It is demonstrated herein that the compound causes a lower-fold decrease in luminescence when the analyte of interest is present than the fold decrease in luminescence when the analyte of interest is absent. The enzymatic luminescence measurement may have an autoluminescence component.

In fact, for the majority of experiments described herein, an increase in the enzymatic luminescence measurement was observed when the compound being tested was present. Because autoluminescence is typically very low, in order to observe a more pronounced effect of the compounds on autoluminescence, autoluminescence was enhanced by adding a detergent, increasing the pH, adding hydrogen peroxide, adding DMSO, adding BSA, or adding sodium hydrosulfite.

Format A Assays were performed in the presence of Steady Glo® reagent (SG) and Stop & Glo® reagent (S+G), (Promega Corporation, Madison Wisconsin). A total reaction volume of 150 µl consisted of:

50 µl F-12 (Ham) media + 1 mg/ml gelatin (with or without enzyme[1])

50 µl S+G (containing substrate[2])

50 µl SG

[1] *Renilla* luciferase enzyme was added to F-12 cell culture media containing 1% gelatin, to a concentration of approximately 2.5ng/50 µl media. Reactions in the absence of *Renilla* luciferase reveal the effect of the compound on autoluminescence while reactions in the presence of enzyme reveal the effect of the compound on *Renilla* luciferase-catalyzed luminescence.

[2] The S+G reagent was prepared as per manufacturer's instructions, with the exception that for these experiments a S+G solvent three times more concentrated than normal was used to resuspend the S+G substrate. Under these conditions, a higher concentration of

coelenterazine in the S+G was needed for substrate to reach saturation conditions.

The compound to be tested was re-suspended in either SG or S+G reagent to a final concentration of SG or S+G of (1X). The compound was added so the final concentration in the 150 µl total volume would be that listed in Table 1 and the reagent was diluted to the a final of 50 µl with water. For controls, the SG or S+G reagent was brought up to 50 µl with water or with the solvent used to dissolve the compound of interest. For example, if a compound needed to be dissolved in DMSO (dimethyl sulphoxide), an equal volume of DMSO was added to the control reaction. Unless otherwise indicated, the compounds were first dissolved in water. The same result can be obtained by adding the compound to be tested directly to the media portion of the reaction instead of to the SG or S+G.

For each concentration of a particular compound, a mixture containing all of the components in sufficient amounts for four reactions (i.e. 200 µl media, 200 µl SG, 200 µl S+G) was assembled. From this mixture, 150 µl was dispensed into triplicate wells on a 96-well plate. Alternatively, reactions were sometimes assembled in each well of the plate by adding each of the 50 µl portions and mixing. The plate was incubated at 22°C and after 5 minutes the luminescence was measured using a Dynex plate luminometer (1 second measurement per well).

Format B Experiments were performed in a reaction volume of 150 µl Matthew's Buffer (referred to herein as MB) as either a standard MB composition or a modified MB composition as described below. As with Format A, reactions with and without *Renilla* luciferase were carried out to observe the effect of the organic compounds on assay sensitivity. In order to be able to add the reaction components such as enzyme, substrate, detergent, and compound to be tested; the reaction was assembled in 3 portions as follows:

50 µl MB	(with or without enzyme[3])
50 µl MB with Coelenterazine	(with or without detergent[4])

50 µl MB (with or without compound[5])

[3] Enzyme was added to 1X MB to a concentration of approximately 2.5 ng/50 µl buffer

[4] Detergents are known to increase the level of autoluminescence. For completeness, the effect of the compounds on autoluminescence and on enzymatic luminescence was evaluated in the presence and absence of detergent.

2X MB was used to make 1X MB without or with detergent at a concentration of 1%Tergitol NP-9, 1% antifoaming agent (0.33% in final reaction). To this portion, coelenterazine was added at a concentration of 180 µM in the version with detergent and of 60 µM in the version without detergent. These levels of substrate are needed to reach saturation conditions.

Note: Other detergents, such as Tween-20 (Sigma Corp., St. Louis, MO) and Zwittergent® 3-08 (CalBiochem, Indianapolis, IN) were found to provide effects similar to those obtained with Tergitol NP-9.

[5] 2X MB was used to make 1X MB with the compound to be tested at various concentrations and water. For controls, 1X MB was made with only water or with water and the addition of the solvent used to solubilize the compound to be tested. For example, if the compound to be tested was dissolved in DMSO, an equal amount of only DMSO was added to the control MB sample.

1X Matthew's buffer standard composition consists of:

100mM potassium phosphate

500mM sodium chloride

1mM ethylenediaminetetraacetic acid (EDTA)

0.1mg/ml bovine serum albumin (BSA)

pH 7.4

The BSA functions as an enzyme stabilizer and, in the standard MB composition, enhances coelenterazine autoluminescence but not to the extent of the autoluminescence generated when detergent is present. In order to observe the autoluminescence enhanced only by the detergent, for the majority of the experiments, BSA was replaced with porcine gelatin as the enzyme stabilizer at a final concentration of 0.15 mg/ml or 0.45 mg/ml. Taking all the variants into account the format can be sub-divided in 4 different versions:

B1 BSA/Detergent

B2 BSA/No Detergent

B3 Gelatin/Detergent

B4 Gelatin/No Detergent

Reactions were carried out in triplicate by adding each of the 50µl portions to microtiter plate wells and mixing. The resulting relative light units generated per well was measured immediately using a Dynex MLX Microtiter plate luminometer or a Wallac 1450 MicroBeta Trilux plate luminometer (1 second/well) or alternatively, the plate was incubated at 22°C and read after 5 minutes in the same fashion.

Results in Table 1 herein below are shown as:

a) fold-decrease in non-enzymatic autoluminescence measurement in the presence of the compound when compared to the absence of the compound and,

b) effect of the compound on enzymatic luminescence measurement in the presence of the compound when compared to control samples lacking only the compound.

For example, a result of "decreased 7.4 fold" indicates that the luminescence measurement in the presence of the compound was 7.4 times less than

the luminescence measurement in the absence of the compound. In all cases, the fold decrease in luminescence not associated with the presence of *Renilla* luciferase (autoluminescence) was greater than the fold decrease in luminescence associated with the presence of *Renilla* luciferase. Thus, the compounds reduce the luminescence not associated with enzymatic activity of the analyte to a greater degree than the luminescence associated with the enzymatic activity of the analyte.

Table 1. See Figure 4 for compound identity.

Format overview:

A Media:Steady Glo:Stop & Glo

B1 Matthew's Buffer with BSA and detergent

B2 Matthew's Buffer with BSA without detergent

B3 Matthew's Buffer with gelatin and detergent

B4 Matthew's Buffer with gelatin without detergent

Compound	mM compound (in final soln)	Format	Fold decrease auto-luminescence	Effect on luminescence
1	33	A	15	No effect
1	316	B2	2	Increased 1.2 fold
1	10	B4	2	Increased 2.1 fold
1	100	B3	290	No effect
1	100	B4	8.5	Increased 1.4 fold
1	50	B3 + 17% DMSO	500	Increased 5 fold
1	50	B4 + 17% DMSO	15	Increased 7 fold
1	3	B3 + 10mM sodium hydrosulfate	21.8	Decreased 7.4 fold
1	3	B4 + 10mM sodium hydrosulfate	2.6	No effect
1	3	B3	120	No effect

1	3	B3 with Tween-20	100	No effect
1	3	B3 with Zwittergent A	6	Increased 1.6 fold
4	10	B3	3	No effect
4	30	B4	500	Decreased 4.6 fold
4	30		3.9	Increased 1.2 fold
5	10		115	Decreased 10 fold
6	32	A + 17% DMSO	65	Decreased 1.5 fold
6	32	B3 + 33% DMSO	660	Increased 1.6 fold
6	32	B4 + 33% DMSO	120	Increased 4.7 fold
2	10	A	100	No effect
2	10	B3	70	No effect
3	33	A	100	Increased 1.2 fold
3	33	B3	545	Increased 1.2 fold
3	33	B4	3.4	Increased 2.3 fold
K SCN	10	B3	55	No effect
K SCN	10	B4	1.7	Increased 1.2 fold
7	30	B3	7	Increased 1.2 fold
7	30	B4	6.2	Increased 2.9 fold
8	100	A	6	No effect
8	30	B3	20	No effect
8	30	B4	3.5	Increased 1.5 fold
9	30	B3	13	Decreased 1.4 fold

9	30	B4	2.5	No effect
9	100	B4	3	Increased 4.7 fold
12	30	B3	2	No effect
12	30	B4	2.3	Increased 1.5 fold
12	100	B4	3.7	Increased 1.8 fold

Example 2. Reduction of Autoluminescence generated by other substrates

In addition to looking at the effect of representative compounds on autoluminescence generated from native coelenterazine (Promega Corporation, Madison Wisconsin) the effect of representative compounds on autoluminescence generated by other substrates was investigated. Coelenterazine analogs N, F, H, HPC, and CP were obtained from Molecular Probes, Eugene Oregon. *Cypridina* luciferin was obtained from NanoLight Technology, Pittsburgh, Pennsylvania. Beetle luciferin was obtained from Promega Corporation, Madison Wisconsin. In order to see a more pronounced effect of the compounds, autoluminescence was enhanced as described in Example 1 by the addition of DMSO or detergent (1%Tergitol NP-9/1%Antifoam®) in Matthew's Buffer. Autoluminescence was also enhanced by addition of H_2O_2 or by raising the pH of the reaction containing native coelenterazine. Experimental conditions are grouped under Format C, sub-divided as follows:

C1 Alternative substrates in DMSO

100 µl per well consisting of:

94 µl DMSO

1 µl substrate at 3 mM (30 µM final substrate concentration)

5 µl of compound dissolved in water or water as the control

C2 Alternative substrates in MB with detergent

100 µl per well consisting of:

94 µl MB with gelatin in place of BSA and detergent

1 µl substrate at 3 mM (30 µM final substrate concentration)

5 µl of compound dissolved in water or water as the control

C3 MB with BSA, pH 9

100 µl per well consisting of:

90 µl MB (standard composition but at pH 9, 30 µM coelenterazine, no detergent present)

5 µl H_2O_2 at 30.7% (1.5% final)

5 µl of compound dissolved in water or water as the control

Reactions were carried out in triplicate by adding each of the components to microtiter plate wells and mixing. The light output was measured immediately using a Dynex MLX Microtiter plate luminometer or a Wallac 1450 MicroBeta Trilux plate luminometer (1 second/well). Results are shown in Table 2.

Table 2

Substrate	mM thiourea Concentration	Format	Fold decrease autoluminescence
native coelenterazine	25	C1	4.8
coelenterazine analog N	25	C1	5
coelenterazine analog F	25	C1	2.6
coelenterazine analog H	25	C1	1.8
coelenterazine analog HPC	25	C1	7.2
coelenterazine analog CP	25	C1	8.7
Cypridina coelenterazine	25	C1	4.2
Beetle luciferin	25	C1 in alkaline environment	3.8
native coelenterazine	25	C2	1100
coelenterazine analog N	25	C2	950
coelenterazine analog F	25	C2	770
coelenterazine analog H	25	C2	720
coelenterazine analog HPC	25	C2	910
coelenterazine analog CP	25	C2	900
Cypridina coelenterazine	25	C2	310
native coelenterazine	50	C3	48

Example 3. Reduction of luminescence generated by chemiluminescent substrates CDP-*Star*® and Luminol

The effect of a representative compound on chemiluminescent reactions containing CDP-*Star*® or Luminol was measured. CDP-*Star*® is a stabilized 1,2 –dioxetane chemiluminescent enzyme substrate, a high energy luminogenic molecule, used in the detection of alkaline phosphatase and alkaline phosphatase conjugates in solution and in membrane-based assays. CDP-*Star*® was obtained from Tropix PE Biosystems, Bedford, Massachusetts. CDP-Star® substrate produces a light signal when it is activated by alkaline phosphatase. Alkaline phosphatase dephosphorylates the substrate, yielding an anion that accumulates due to its long half-life. Since the ultimate light production involves decomposition of the anion, a delay precedes constant signal output, resulting in a glow of light that lasts for hours to days. Luminol (5-Amino-2,3-didydro-1,4-phthalazinedione) was obtained from Sigma, St. Louis, Missouri. Luminol is a widely used chemiluminescent reagent, that luminesces upon oxidation. Experimental conditions are grouped under Format D, sub-divided as follows:

D1 CDP-*Star*® + thiourea in water

In microtiter plate wells, various amounts of CDP-*Star*® reagent as listed below were mixed with the representative compound in water (or water alone as control) and measured in a luminometer as previously described (1 second/well).

50% 50 µl CDP-*Star*® + 50 µl 0.5 M thiourea (250 mM CDP-*Star*® final conc.)

75% 75 µl CDP-*Star*® + 25 µl 0.5 M thiourea (125 mM CDP-*Star*® final conc.)

95% 95 µl CDP-*Star*® + 5 µl 0.5 M thiourea (25 mM CDP-*Star*® final conc.)

D2 CDP-*Star*® undiluted

Thiourea was dissolved directly into CDP-*Star*® reagent at a concentration of 10 mM. Control reactions contained CDP-*Star*® reagent alone (100 µl per well) and light output was measured on a MLX Microtiter plate

luminometer or a Wallac 1450 MicroBeta Trilux plate luminometer (1 second read per well).

Parallel wells containing 0.28 pg alkaline phosphatase were also measured to monitor the CDP-Star's integrity and activity in these conditions.

D3 Luminol

When a solution containing Luminol comes in contact with H_2O_2, a chemiluminescent reaction occurs. The effect of thiourea on this Luminol reaction was measured on the following reactions assembled in a microtiter plate:

50 µl Luminol solution[6]

45 µl 0.0015% or 0.00015% H_2O_2

5 µl 0.5 M thiourea (25 mM final conc.) or water as control

[6] Luminol solution per 100 ml:

0.4 gm sodium carbonate

0.02 gm luminol

2.4 gm sodium bicarbonate

0.05 gm ammonium carbonate

0.04 gm copper (II) sulfate pentahydrate

distilled water to 100ml

pH 9.0

The resulting luminescence was immediately measured using a Dynex MLX Microtiterplate luminometer or a Wallac 1450 MicroBeta Trilux plate luminometer (1 sec read per well). The results are listed in Table 3. The data demonstrate that thiourea acts on both CDP-*Star*® and Luminol chemiluminescence reactions to decrease autoluminescence.

Table 3

Substrate	mM thiourea conc. (final)	Format	Fold decrease luminescence
CDP-*Star*	250	D1 50%	40
CDP-*Star*	125	D1 75%	6
CDP-*Star*	25	D1 95%	2
CDP-*Star*	10	D2	1.6*
Luminol	25	D3 0.0015% H2O2	150,000
Luminol	25	D3 0.00015% H2O2	3840

*A parallel reaction was conducted containing Alkaline Phospatase (AP) to monitor the effect of thiourea on CDP-*Star*® stability. The reaction containing AP also decreased the luminescence output but at a lesser magnitude than that of the CDP-*Star*® alone. The AP reaction decreased 1.4 fold in the presence of thiourea.

Example 4. Effect of pH on coelenterazine derived autoluminescence

The following experiment was performed to determine the ability of thiourea to reduce autoluminescence at various pH values.

Matthew's buffer was made at a 2X concentration (standard formulation as described in Example 1) and divided in several aliquots. Aliquots were adjusted from pH 4 to pH 9 in one pH unit increments. For each pH, versions of the buffer were made with or without 1%Tergitol NP-9/1%antifoaming agent (referred to as "detergent") and with or without 3 mM thiourea. Coelenterazine was added to a concentration of 180 mM for the version with detergent, and to a concentration of 60 mM for the version without detergent. The buffers were then brought to a final 1X concentration with water.

A 150 µl aliquot of each reaction was dispensed in triplicate into microtiter plate wells and the plate was incubated at 22°C. After 5 minutes the luminescence was measured using a plate luminometer as previously described (1sec per well) and the results are shown in the following tables:

Matthew's Buffer with Detergent

	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
Luminescence without thiourea (relative light units)	1.45	3.78	56	137.5	224.7	361.1
Luminescence with 3mM thiourea (relative light units)	.0375	.04625	.21	.71	1.62	1.77
Fold Reduction	38.3	81.7	266.8	193.6	138.4	204.4

Matthew's Buffer without Detergent

	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
Luminescence without thiourea (relative light units)	0.0925	0.2	0.78	3.63	39.62	56.36
Luminescence with 3mM thiourea (relative light units)	0.025	0.024	0.063	0.259	1.52	1.785
Fold Reduction	3.7	8.4	12.4	14	26.1	31.6

These results demonstrate that increasing the pH of the buffer increases coelenterazine autoluminescence. The addition of 3 mM thiourea effectively decreases autoluminescence even at high pHs.

Example 5. Reduction of Autoluminescence in Whole Cell Assay

To determine the effect of organic compounds on cell viability and to determine the ability of such compounds to reduce autoluminescence in the presence of living cells, the following experiment was performed. Human embryonic kidney

cells (293, ATCC, Rockville, MD) were used to generate a cell line that stably expresses the firefly luciferase (Luc+) gene. This stable cell line was made using the pCl-neo vector (Promega Corporation, Madison Wisconsin, USA) and inserting the *Luc+* gene between the *Xba* I and *Sal* I sites. The stable cell line was prepared using standard methods and the transformed cells were grown in wells of a microtiter plate in the presence of DMEM medium containing 10% FBS and 0.15 mg/ml G418. For experimental purposes, duplicate plates of cells were prepared using 96 well microtiter plates. One plate was used to examine viability, and the other plate was used to examine the effect on autoluminescence.

To examine the effect of the organic compounds in cell viability, the media was removed from the cells and replaced with media containing the substrate for firefly luciferase, beetle luciferin, and the organic compounds at various concentrations. Passive diffusion of luciferin across the cell membrane together with the ATP oxygen and luciferase enzyme already contained within the cell, results in light production. Whereas, in compromised or damaged cells, intracellular ATP concentration is rapidly depleted, decreasing the firefly luminescence. The level of luminescence was compared to controls containing only luciferin to identify the effect of the compounds, if any, on light output as an indicator of cell viability.

To determine the ability of the organic compounds to reduce autoluminescence, the media was removed from the cells and replaced with media containing the compounds at various concentrations and coelenterazine. Since there is no *Renilla* luciferase enzyme being expressed in these cells, the only luminescence observed is autoluminescence. The level of autoluminescence was compared to controls containing only coelenterazine to identify the effect of the compounds on reducing autoluminescence.

Half of the microtiter plate contained no cells. To these wells, the same reagents were added as to the cell counterpart to measure cell-independent luminescence (i.e. background luminescence). The reagents (media with luciferin or coelenterazine, with or without compounds at various concentrations) were prepared as follows:

a) Reagent to examine firefly luminescence (cell viability)

Luciferin substrate available from Promega Corporation, Madison WI, USA was initially prepared in 10mM sodium phosphate buffer, pH 7.4 as a 100mM stock. This luciferin stock was used to make DMEM solution containing a final concentration of 2mM luciferin.

Thiourea and 1-allyl-3-(2-hydroxyethyl)-2-thiourea were dissolved directly in this solution (DMEM/luciferin) to a final concentration of 30mM. These were subsequently diluted to contain final compound concentrations of 10mM and 1mM.

Another compound 6-aza-thio-thymidine was dissolved in DMSO as a 750mM stock. The 6-aza-thio-thymidine was subsequently added to the DMEM/luciferin reagent at a final concentration of 30mM, 10 mM, and 1 mM, while maintaining a final DMSO concentration of 4%. A DMEM/luciferin reagent was used as the control with which to compare the effect of the compound and was also made to contain a final DMSO concentration of 4%.

Since it is believed that DMSO may help organic molecules permeat cell membranes, an additional control was included that consisted of thiourea (10 mM) reconstituted in DMSO (4%).

b) Reagent to examine reduction in autoluminescence

Coelenterazine substrate was initially dissolved in Stop & Glo® Reagent Solvent (both available from Promega Corporation, Madison WI, USA) as a 30mM stock. This coelenterazine stock was used to make DMEM media containing 0.6mM coelenterazine as the final concentration. DMEM/coelenterazine reagents were made in a similar fashion as the DMEM/luciferin reagents described in a).

The cell culture medium was removed from the cells and replaced with medium containing substrate (+/- compound) and luminescence was measured immediately. All luminescent values obtained from wells containing cells were background subtracted using the corresponding luminescent values from those wells that did not contain cells. Fold reduction in autoluminescence was calculated by

dividing the background-subtracted autoluminescence in minus compound controls by the background-subtracted autoluminescence containing the compounds.

Results for representative compounds are shown in the following Table.

Compound	Concentration	Additives to Media?	Effects on firefly luminescence	Fold Decrease in Autoluminescence
Thiourea	30 mM	No	Yes, decreases	40
Thiourea	10 mM	No	No	16
Thiourea	1 mM	No	No	3.4
Thiourea	10 mM	4% DMSO	No	12
1-Allyl-3-(2-hydroxyethyl)-2-thiourea	30 mM	No	Yes, decreases	35
1-Allyl-3-(2-hydroxyethyl)-2-thiourea	10 mM	No	Yes, decreases	12
1-Allyl-3-(2-hydroxyethyl)-2-thiourea	1 mM	No	No	3.5
6-aza-thio-thymidine	30 mM	4% DMSO	Yes, decreases	525
6-aza-thio-thymidine	10 mM	4% DMSO	No	23
6-aza-thio-thymidine	1 mM	4% DMSO	No	3

These results demonstrate that these compounds can be used to reduce autoluminescence in luminescent assays employing whole cells without significantly decreasing cell viability.

All publications, patents, and patent documents are incorporated by reference herein, as though individually incorporated by reference. The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications can be made while remaining within the spirit and scope of the invention.